



## Association of Genetic Variation with Keratoconus

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# Genetic variation on chromosome 11 is associated with keratoconus

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3000 words.

37 **Key points**

38 **Question** Which genetic loci associate with keratoconus?

39 **Findings** In this case-control genome-wide association study, with three independent replication cohorts, a  
40 locus containing multiple variants across six protein-coding genes on chromosome 11 was associated with  
41 keratoconus. Several of these genes are likely involved in apoptotic pathways.

42 **Meaning** This study of patients with keratoconus demonstrates a potential role of genes involved in  
43 apoptotic pathways.

44

## 45 Abstract

46 **Importance** Keratoconus is a condition in which the cornea progressively thins and protrudes in a conical  
47 shape, severely affecting refraction and vision. It is a major indication for corneal transplantation. To  
48 discover new genetic loci associated with keratoconus and to better understand the causative mechanism of  
49 this disease, we performed a genome-wide association study (GWAS) on patients with keratoconus.

50 **Objective** To identify keratoconus genetic susceptibility regions in the human genome.

51 **Design** The discovery cohort was genotyped using the Illumina HumanCoreExome single nucleotide  
52 polymorphism (SNP) array. Following quality control and data cleaning, genotypes were imputed against the  
53 1000 Genomes Project reference panel (Phase III, version 5) and association analysis was completed using  
54 PLINK. SNPs with  $P < 1 \times 10^{-6}$  were assessed for replication in three additional cohorts.

55 **Setting** Eye clinics in Australia, the USA, and Northern Ireland.

56 **Participants** The discovery cohort comprised 522 Australian keratoconus cases and 655 Australian  
57 controls. Controls were drawn from the Blue Mountains Eye Study and normal individuals previously  
58 recruited as controls for a study of glaucoma. Replication cohorts were a previous keratoconus GWAS  
59 dataset from the USA (222 cases, 2927 controls); 331 cases and 229 controls from Australia and Northern  
60 Ireland (AUS+NI), and an Australian case–control cohort (VIC, 265 cases, 702 controls).

61 **Main Outcomes and Measures** Associations between keratoconus and 6,252,612 genetic variants were  
62 estimated using logistic regression, were adjusting for ancestry using the first three principal components.

63 **Results** Two novel loci reached genome-wide significance (defined as  $P < 5 \times 10^{-8}$ ), with  $P = 7.46 \times 10^{-9}$  at  
64 rs61876744 in *PNPLA2* on chromosome 11 and  $P = 6.35 \times 10^{-12}$  at rs138380 2.2kb upstream of *CSNK1E* on  
65 chromosome 22. One additional locus was identified with  $P < 10^{-6}$ , in *MAML2* on chromosome 11. The  
66 novel locus in *PNPLA2* reached genome-wide significance in meta-analysis of all four cohorts ( $P = 2.45 \times$   
67  $10^{-8}$ ).

68 **Conclusions and Relevance** In this relatively large keratoconus GWAS, we identified a genome-wide  
69 significant locus for keratoconus in the region of *PNPLA2* on chromosome 11.

70

## 71    **Introduction**

72    Keratoconus is characterized by progressive thinning of the cornea, the clear tissue at the front of the eye.  
73    Asymmetrical bulging and conical protrusion of the cornea leads to extreme refractive error (myopia and  
74    irregular astigmatism) causing severe visual impairment.<sup>1</sup> Keratoconus is relatively common, with a reported  
75    prevalence of around 55 per 100,000 in white populations<sup>1</sup> and up to 229 per 100,000 in Asian populations.<sup>2</sup>  
76    Due to recent advances in diagnostic imaging the true incidence and prevalence of keratoconus may be 5 to  
77    10 times higher than previously reported.<sup>3</sup> The etiology of keratoconus is poorly understood. Links have  
78    been made to eye rubbing and atopy, but no direct causative connection has been established.<sup>4</sup> Various  
79    biochemical pathways may be involved, including oxidative stress, apoptosis, and disruption to extracellular  
80    matrix turnover.<sup>5</sup>

81    Although many cases of keratoconus present as sporadic, there is a well-recognised genetic component to the  
82    disease. The estimated prevalence in relatives of keratoconus patients is 3.34% (95% CI: 3.22–3.46%),  
83    which is 15–67 times higher than in the general population.<sup>6</sup> In addition, more than 20 syndromes are  
84    associated with keratoconus, including Down syndrome, Leber congenital amaurosis, and several connective  
85    tissue disorders.<sup>7</sup> Linkage studies have identified at least 16 loci for keratoconus;<sup>8,9</sup> however, the causative  
86    genes and variants have remained elusive. Common variants in the *DOCK9*<sup>10</sup> and *LOX*<sup>11</sup> genes have been  
87    implicated as well as rare mutations in *MIR184*,<sup>12,13</sup> although these loci have not been broadly replicated.  
88    Genome-wide association studies (GWAS) have implicated several loci. Variation in the promoter region of  
89    the *HGF* gene<sup>14,15</sup> and upstream of *RAB3GAP1*<sup>16,17</sup> have both been associated in multiple independent  
90    studies. Further, a GWAS for central corneal thickness (CCT) identified loci that are also associated with  
91    keratoconus, including *RXRA–COL5A1*, *FOXO1* and *FNDC3B*,<sup>18</sup> and more recently a suggestive association  
92    at *DCN*.<sup>19</sup>

93    We present findings from a GWAS of 522 patients with keratoconus, a relatively large sample for this  
94    complex disease. We show association and independent replication at the *PNPLA2* region with keratoconus.

95

## 96 **Methods**

### 97 **Study design**

98 We report a study of 4 independent Caucasian cohorts of patients with keratoconus. The first case–control  
99 cohort used for the discovery phase comprised 522 keratoconus patients and 655 controls. All single  
100 nucleotide polymorphisms (SNPs) with  $P < 1 \times 10^{-6}$  were looked up in imputed genotypes from a previously  
101 published GWAS study of 222 keratoconus patients and 3324 controls from the USA. In addition, 27 SNPs  
102 were genotyped in an independent replication cohort of 331 cases and 229 controls from Australia and  
103 Northern Ireland (AUS+NI), and in an additional 265 cases and 702 controls from Victoria, Australia (VIC).  
104 The demographics for each cohort are given in Table 1.

### 105 **Discovery cohort**

106 The protocol was approved by the Southern Adelaide Clinical Human Research Ethics Committee (HREC)  
107 and the HREC of the Royal Victorian Eye and Ear Hospital and the Health and Medical HREC of the  
108 University of Tasmania. All participants gave written informed consent and the study conformed to the  
109 tenets of the Declaration of Helsinki. Participants with keratoconus were ascertained through the eye clinic  
110 of Flinders Medical Centre, Adelaide; optometry and ophthalmology clinics in Adelaide and Melbourne; or  
111 an Australia-wide invitation to members of Keratoconus Australia, a community-based support group for  
112 patients. Clinical data were obtained from the participants' eye care practitioner.

113 The diagnosis of keratoconus was based on both clinical examination and videokeratography pattern analysis  
114 as described previously.<sup>20</sup> Clinical examination included slit-lamp biomicroscopy, cycloplegic retinoscopy,  
115 and fundus evaluation. Slit-lamp biomicroscopy was used to identify stromal corneal thinning, Vogts' striae,  
116 or a Fleischer ring. Retinoscopy examination was performed on a fully dilated pupil to determine the  
117 presence or absence of retro-illumination signs of keratoconus, such as the oil droplet sign and scissoring of  
118 the red reflex. Videokeratography evaluation was performed on each eye using the Orbscan (Orbtek/Bausch  
119 & Lomb, Salt Lake City, UT, USA). Patients were classified as having keratoconus if they had at least one  
120 clinical sign of keratoconus and a confirmatory videokeratography.<sup>21</sup> A history of penetrating keratoplasty  
121 performed because of keratoconus was also sufficient for inclusion as a case. Patients with syndromic forms

122 of keratoconus were excluded, and if multiple individuals from the same family presented, only one was  
123 included.

124 Controls were obtained from the Australian cohort previously described in a GWAS for age-related macular  
125 degeneration (AMD) from the International AMD Genomics Consortium<sup>22</sup> and have been described in detail  
126 previously. For the current analysis, data from 676 Australian unaffected controls (including 465 from the  
127 Blue Mountains Eye Study<sup>23</sup> and 211 normal individuals previously recruited as controls for a study of  
128 glaucoma<sup>24</sup>) were combined as controls for keratoconus. Related individuals and those who did not pass all  
129 sample QC for the AMD GWAS were excluded.

130 DNA for cases and controls was extracted from whole blood using the QiaAMP DNA Maxi kit (Qiagen,  
131 Hilden, Germany).

## 132 **Genotyping and data quality control**

133 Cases were genotyped for 551,839 variants using the HumanCoreExome array (HumanCoreExome-24v1-  
134 1\_A, Illumina, San Diego, CA, USA). For the controls, genotypes of 569,645 variants were generated with a  
135 customized Illumina HumanCoreExome array ("HumanCoreExome\_Goncalo\_15038949\_A") as described  
136 previously.<sup>22</sup>

137 Quality control was carried out according to the protocol described by Anderson et al<sup>25</sup>, modified as follows.  
138 Reverse and ambiguous strand SNPs were detected using snpflip (<https://github.com/biocore-ntnu/snpflip>,  
139 accessed March 24, 2017) and flipped or excluded. Only SNPs common to both arrays were included.

140 Individuals with discordant sex information, missing genotype rate >0.05, or heterozygosity more than 3  
141 standard deviations from the mean were excluded. Related individuals were detected by calculating pairwise  
142 identity by descent (IBD), and the individual with the lower genotyping rate in any pair with IBD >0.185  
143 was removed. Ancestry outliers were identified by principal component analysis (PCA) using  
144 EIGENSTRAT,<sup>26</sup> and removed. Markers were excluded if they had missing genotype rate >3%, significantly  
145 different missing data rates between cases and controls, minor allele frequency (MAF) <0.01, or deviated  
146 significantly ( $P < 10^{-5}$ ) from Hardy–Weinberg equilibrium. Following all exclusions, there were 522 cases  
147 and 655 controls genotyped for 264,115 common platform SNPs.

## 148    **Genomic imputation and association analysis**

149    We phased autosomal genotype data using Eagle (version 2.3.5)<sup>27</sup> and then imputed genotypes on the basis  
150    of the EUR subset of the 1000 Genomes Project reference panel (Phase III, version 5)<sup>28</sup> using Minimac3  
151    (version 2.0.1).<sup>29</sup> We excluded indels, SNPs within 5bp of an indel, rare variants (MAF <0.01), and variants  
152    with poor imputation quality ( $R^2 < 0.8$ ). This filtering yielded a total of 6,252,612 quality-controlled variants,  
153    including 250,964 genotyped variants. Association analysis was performed on most-likely genotypes under a  
154    logistic regression model using PLINK (version 1.90)<sup>30</sup> using the first three principal components as  
155    covariates. A  $P$  value less than  $5 \times 10^{-8}$  was considered significant.

## 156    **Replication and meta-analysis**

157    The USA cohort has been previously described.<sup>18</sup> Briefly, clinically affected Caucasian keratoconus cases ( $n$   
158    = 240) were enrolled into the GWAS<sup>31</sup> as a part of the longitudinal videokeratography and genetic study at  
159    the Cornea Genetic Eye Institute.<sup>6</sup> After removing samples with poor genotyping quality, 222 samples were  
160    included in the analysis. Caucasian controls ( $n = 3324$ ) were obtained from the Cardiovascular Health Study  
161    (CHS), a population-based cohort study of risk factors for cardiovascular disease and stroke in adults 65  
162    years of age or older, recruited at 4 field centres.<sup>32,33</sup> CHS was approved by the Institutional Review Board at  
163    each recruitment site, and subjects provided informed consent for the use of their genetic information.  
164    African-American CHS participants were excluded from analysis due to insufficient number of ethnically-  
165    matched cases. Participants did not have an eye examination to exclude keratoconus. The samples included  
166    in the analysis were from self-reported Caucasians. Outliers detected by PCA were excluded, and the  
167    analysis was adjusted for the top 3 principal components.

168    IMPUTE version 2.3.0 was used to perform imputation of the genotyping data from 370K BeadChip arrays  
169    (Illumina) in keratoconus patients and CHS Caucasian controls using 1000 Genomes Phase I data as the  
170    reference panel. All SNPs with  $P < 10^{-6}$  identified in the discovery analysis were looked up and extracted,  
171    with the exception of 4 SNPs at the *CSNK1E* locus that were not imputed in the USA study.

172    SNPs with  $P < 10^{-6}$  in the discovery cohort were selected for genotyping in additional replication cohorts  
173    using the MassARRAY® System (Agena Bioscience, San Diego, CA, USA) by the Australian Genome  
174    Research Facility. SNPs were chosen from each locus that were compatible with the assay design, with



175 preference given to SNPs with the smallest  $P$  value in the discovery cohort. Twenty-seven SNPs were  
176 genotyped in 186 additional cases recruited under the same protocol as the discovery cohort as well as in 145  
177 patients from Northern Ireland, described previously,<sup>14</sup> for a total of 331 cases. Cases were compared to 229  
178 unaffected examined controls, consisting of 84 individuals from the Blue Mountains Eye Study not included  
179 in the discovery cohort and 145 older individuals recruited from nursing homes in the Launceston area of  
180 Tasmania, Australia. All controls underwent a thorough ocular examination and keratoconus was excluded.  
181 These SNPs were also genotyped in a replication cohort from Melbourne, Victoria, consisting of 265 cases  
182 and 702 examined controls described previously.<sup>15</sup>  
183 Association was assessed in each cohort individually using logistic regression. Meta-analysis of results from  
184 discovery and replication cohorts was performed using METAL.<sup>34</sup>

## 185 **Functional annotation of associated variants**

186 The lead SNP at the novel locus on chromosome 11, rs61876744, was queried in HaploReg V4.1  
187 (<https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php>; accessed 28 May 2019),<sup>35</sup> including data  
188 from the Genotype–Tissue Expression (GTEx) pilot analysis.<sup>36</sup> Genes in the associated region were assessed  
189 for ocular tissue expression using the Ocular Tissue Database (<https://genome.uiowa.edu/otdb/>)<sup>37</sup> and for  
190 differential expression between corneas from keratoconus and myopia patients in a previously published  
191 study.<sup>38</sup>

## 193 **Results**

### 194 **Genome-wide association testing of the discovery cohort**

195 Genome-wide association analysis was conducted in the discovery cohort (Figure 1, eFigure 1 in the  
196 Supplement). The genomic inflation factor was  $\lambda_{1000} = 1.023$  and all included samples were of European  
197 ancestry (eFigure 2 in the Supplement). Two novel loci reached genome-wide significance (defined as  $P < 5$   
198  $\times 10^{-8}$ ), with  $P = 7.46 \times 10^{-9}$  at rs61876744 in the *PNPLA2* gene on chromosome 11 and  $P = 6.35 \times 10^{-12}$  at  
199 rs138380 2.2kb upstream of the *CSNK1E* gene on chromosome 22. One additional locus was identified with  
200  $P < 10^{-6}$ , in the *MAML2* gene on chromosome 11 (rs10831500,  $P = 3.91 \times 10^{-7}$ ). Association results for all

201 SNPs with  $P < 10^{-6}$  are shown in eTable 1 in the Supplement. Locus-specific plots for all 3 loci are shown in  
202 eFigure 3 in the Supplement.

203 The locus on chromosome 11 showing genome-wide significance included 25 SNPs with  $P < 10^{-6}$  spanning  
204 6 protein-coding genes (*CEND1*, *SLC25A22*, *PANO1*, *PIDD1*, *RPLP2* and *PNPLA2*) and multiple RNA  
205 genes.

206 Because previously-reported keratoconus risk loci were initially identified in GWAS for CCT, we assessed  
207 each of the loci reaching suggestive significance in the current analysis in our previously reported meta-  
208 analysis for CCT. As shown in Table 2, only the *MAML2* locus shows nominal association with CCT. We  
209 also looked up the lead SNPs from our CCT meta-analysis in the results from our discovery cohort (eTable 2  
210 in the Supplement). SNPs at *FNDC3B*, *MPDZ*, and *SMAD3* show nominal association in our analysis.

### 211 **Association testing of the replication cohorts**

212 All SNPs with  $P < 10^{-6}$  identified in the discovery cohort were analysed in the previously generated GWAS  
213 data for the USA cohort, and 27 SNPs compatible with a single assay design were genotyped in the AUS+NI  
214 and VIC replication cohorts. (Table 3 and eTable 3 in the Supplement).

215 Multiple SNPs in both novel loci at *PNPLA2* and *MAML2* showed association with  $p < 0.05$  in the USA  
216 cohort. None of the SNPs in the *CSNK1E* locus reached significance, although all showed the same direction  
217 of association as in the discovery cohort. None of the 3 loci reached significance in either the AUS+NI or the  
218 VIC replication cohort, but most SNPs showed the same direction of association as the discovery cohort.  
219 MAFs for cases and controls in each cohort are given in eTable 3 in the Supplement.

### 220 **Meta-analysis**

221 Meta-analysis of the combined data from the discovery and all 3 replication cohorts found 12 SNPs at the  
222 novel *PNPLA2* locus to be associated with keratoconus at genome-wide significance ( $P < 5 \times 10^{-8}$ ). The  
223 *MAML2* locus on chromosome 11 showed suggestive association with  $P = 3.83 \times 10^{-6}$  at rs10831500. The  
224 *CSNK1E* locus on chromosome 22 reached  $P = 3.18 \times 10^{-4}$  at rs138378. For all 3 loci, there were other SNPs  
225 with smaller  $P$  values on meta-analysis, but these did not include data for all replication cohorts (eTable 3 in  
226 the Supplement).

## 227    **Functional annotations of novel associated loci**

228    At the significant locus on chromosome 11, the lead SNP rs61876744 is located in the second intron of the  
229    *PNPLA2* gene (NM\_020376.3). The associated region extends for around 40kbp encompassing multiple  
230    transcripts. The *PNPLA2* gene is highly expressed in all eye tissues assessed in the ocular tissue database,  
231    including cornea, as are other protein-coding genes at this locus (eTable 4 in the Supplement). A recent study  
232    compared gene expression in corneal epithelium from keratoconus and myopia patients,<sup>38</sup> and found that  
233    *PNPLA2* and *PIDD1* were differentially expressed with a false discovery rate (FDR) < 0.05 (eTable 4 in the  
234    Supplement) while *RPLP2* and *CSNK1E* are significant at FDR < 0.1.

235    HaploReg identified 7 SNPs in strong linkage disequilibrium with the lead SNP (rs61876744), and all report  
236    an eQTL for an antisense RNA transcript *AP006621* (multiple transcripts 1–8) in multiple tissues assessed in  
237    GTex (eTable 5 in the Supplement) where the more common allele, C, is associated with increased transcript  
238    levels (eFigure 4 in the Supplement). This RNA gene is not represented in the ocular tissue database. A  
239    similar trend is seen in GTex for the *PIDD1* gene in sun exposed skin (eFigure 4 in the Supplement).

## 240    **Discussion**

241    This study has identified a candidate locus for keratoconus on chromosome 11 that shows replication in the  
242    USA data and consistent direction of association in the other cohorts. The lead SNP is located in an intron of  
243    *PNPLA2*. This gene encodes Patatin-like phospholipase domain-containing protein 2, which catalyzes the  
244    initial step in triglyceride hydrolysis. The relevance of this pathway to keratoconus is not obvious, but it is  
245    well known that the closest gene to an association signal is not necessarily the causative gene. At least 4  
246    other protein coding genes at this locus are also expressed in the cornea, and RNA coding genes are also  
247    annotated in the region. There is a strong eQTL signal of the lead SNP rs61876744 for an antisense RNA  
248    gene, *AP006621.8* located on the opposite strand to the protein coding *PNPLA2* gene. The antisense RNA  
249    *AP006621* transcripts may have a role in regulating *PNPLA2* or other genes at this locus and elsewhere. The  
250    minor allele at rs61876744, T, is associated with reduced risk of keratoconus and with reduced expression of  
251    *AP006621* in many tissues. This suggests that overexpression of *AP006621* may destabilise corneal  
252    structures. Oxidative stress and apoptosis have been suggested as part of the pathogenesis of keratoconus<sup>5</sup>  
253    and sun (or UV light) exposure is known to trigger oxidative stress and DNA damage pathways.<sup>39</sup> Several

254 genes at this locus likely play a role in apoptotic pathways, including *PPID* (p53-induced death domain  
 255 protein 1) and *PANO1* (proapoptotic nucleolar protein 1).

256 The chromosome 11 locus overlaps with a previously reported (although not genome-wide significant)  
 257 association signal for Fuchs Endothelial Corneal Dystrophy (FECD).<sup>40</sup> The lead SNP in the FECD GWAS is  
 258 rs12223324 in the *PDDC1* gene, upstream of *PNPLA2*. This SNP does not reach significance in our  
 259 keratoconus GWAS, ( $P = 2.02 \times 10^{-5}$ ). It is not known how this locus might lead to FECD, but the overlap  
 260 of genetic association with keratoconus is intriguing, given both diseases affect the cornea. Although rare,  
 261 there are reports in the literature of patients with both FECD and keratoconus.<sup>41,42</sup> The participants in the  
 262 current study do not have FECD and thus this disease does not account for the association observed here.

263 Although we observed the strongest association in the discovery cohort at the *CSNK1E* locus, this result was  
 264 not replicated. The signal appears to be driven by a single genotyped SNP that has influenced the imputation  
 265 of a surrounding LD block (see eTable 1; eFigure 3C in the Supplement). The signal at the *MAML2* locus is  
 266 supported by the USA replication cohort. Further replication of these loci is required before any firm  
 267 conclusions can be drawn.

268 All control cohorts used in this study were older than the case cohorts, thus it will be important to assess  
 269 these loci for age-related effects in future studies. Batch effects are a potential problem in an analysis where  
 270 cases and controls are genotyped separately; however, the low inflation factor seen in our analysis reassures  
 271 us that batch effects are unlikely to be having a major impact.

272 Previous GWAS for keratoconus (using a subset of cases involved in the current study) reported  
 273 *RAB3GAP1*<sup>31</sup> and a region upstream of the *HGF*,<sup>14</sup> although neither study reached genome-wide  
 274 significance. The lead SNPs at these loci reach  $P$  values of 0.066 and 0.038 respectively in the current  
 275 discovery cohort, suggesting these loci are not major contributors in this better powered study. Previously  
 276 reported genome-wide significant keratoconus associated loci (*RXRA–COL5A1*, *FOXO1* and *FNDC3B*) are  
 277 also associated with CCT. The current findings suggest that mechanisms other than susceptibility to a thinner  
 278 cornea may also be at play in the genetic risk of keratoconus.

279 In summary, we have identified a locus for keratoconus on chromosome 11. The lead SNP is in an intron of  
 280 the *PNPLA2* gene and is an eQTL for a long non-coding RNA *AP006621.8*. We also suggest loci near

281 *MAML2* and *CSNK1E* that require further replication. It is very likely that additional risk loci exist for  
282 keratoconus, and larger studies will be needed to identify them.

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312 *Study concept and design:* Burdon, Craig, Baird, Bykhovskaya, MacGregor, Willoughby.

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322 **Conflict of Interest:**

323 None of the authors has any conflicts of interest to disclose.

324

## 325    **References**

- 326    1.        Rabinowitz YS. Keratoconus. *Surv Ophthalmol*. 1998;42:297-319.
- 327    2.        Pearson AR, Soneji B, Sarvananthan N, Sandford-Smith JH. Does ethnic origin influence the  
328        incidence or severity of keratoconus? *Eye*. 2000;14:625-628.
- 329    3.        Godefrooij DA, de Wit GA, Uiterwaal CS, Imhof SM, Wisse RP. Age-specific incidence and  
330        prevalence of keratoconus: A nationwide registration study. *Am J Ophthalmol*. 2017;175:169-172.
- 331    4.        Zadnik K, Barr JT, Edrington TB, et al. Baseline findings in the Collaborative Longitudinal  
332        Evaluation of Keratoconus (CLEK) Study. *Invest Ophthalmol Vis Sci*. 1998;39:2537-2546.
- 333    5.        Wojcik KA, Blasiak J, Szaflik J, Szaflik JP. Role of biochemical factors in the pathogenesis of  
334        keratoconus. *Acta Biochim Pol*. 2014;61:55-62.
- 335    6.        Wang Y, Rabinowitz YS, Rotter JI, Yang H. Genetic epidemiological study of keratoconus:  
336        Evidence for major gene determination. *Am J Med Genet*. 2000;93:403-409.
- 337    7.        Nowak DM, Gajecka M. The genetics of keratoconus. *Middle East Afr J Ophthalmol*. 2011;18:2-6.
- 338    8.        Abu-Amero KK, Al-Muammar AM, Kondkar AA. Genetics of keratoconus: Where do we stand? *J*  
339        *Ophthalmol*. 2014;2014:641708.
- 340    9.        Burdon KP, Vincent AL. Insights into keratoconus from a genetic perspective. *Clin Exp Optom*.  
341        2013;96:146-154.
- 342    10.       Czugala M, Karolak JA, Nowak DM, et al. Novel mutation and three other sequence variants  
343        segregating with phenotype at keratoconus 13q32 susceptibility locus. *Eur J Hum Genet*.  
344        2012;20:389-397.
- 345    11.       Bykhovskaya Y, Li X, Epifantseva I, et al. Variation in the lysyl oxidase (*LOX*) gene is associated  
346        with keratoconus in family-based and case-control studies. *Invest Ophthalmol Vis Sci*. 2012;53:4152-  
347        4157.
- 348    12.       Hughes AE, Bradley DT, Campbell M, et al. Mutation altering the miR-184 seed region causes  
349        familial keratoconus with cataract. *Am J Hum Genet*. 2011;89:628-633.
- 350    13.       Lechner J, Bae HA, Guduric-Fuchs J, et al. Mutational analysis of *MIR184* in sporadic keratoconus  
351        and myopia. *Invest Ophthalmol Vis Sci*. 2013;54:5266-5272.

- 352 14. Burdon KP, Macgregor S, Bykhovskaya Y, et al. Association of polymorphisms in the hepatocyte  
353 growth factor gene promoter with keratoconus. *Invest Ophthalmol Vis Sci.* 2011;52:8514-8519.
- 354 15. Sahebjada S, Schache M, Richardson AJ, Snibson G, Daniell M, Baird PN. Association of the  
355 hepatocyte growth factor gene with keratoconus in an Australian population. *PloS one.*  
356 2014;9:e84067.
- 357 16. Bae HA, Mills RA, Lindsay RG, et al. Replication and meta-analysis of candidate loci identified  
358 variation at *RAB3GAP1* associated with keratoconus. *Invest Ophthalmol Vis Sci.* 2013;54:5132-  
359 5135.
- 360 17. Li X, Bykhovskaya Y, Haritunians T, et al. A genome-wide association study identifies a potential  
361 novel gene locus for keratoconus, one of the commonest causes for corneal transplantation in  
362 developed countries. *Hum Mol Genet.* 2012;21:421-429.
- 363 18. Lu Y, Vitart V, Burdon KP, et al. Genome-wide association analyses identify multiple loci  
364 associated with central corneal thickness and keratoconus. *Nat Genet.* 2013;45:155-163.
- 365 19. Iglesias AI, Mishra A, Vitart V, et al. Cross-ancestry genome-wide association analysis of corneal  
366 thickness strengthens link between complex and Mendelian eye diseases. *Nat Commun.*  
367 2018;9:1864.
- 368 20. Burdon KP, Coster DJ, Charlesworth JC, et al. Apparent autosomal dominant keratoconus in a large  
369 Australian pedigree accounted for by digenic inheritance of two novel loci. *Hum Genet.*  
370 2008;124:379-386.
- 371 21. Rabinowitz YS. Videokeratographic indices to aid in screening for keratoconus. *J Refract Surg.*  
372 1995;11:371-379.
- 373 22. Fritsche LG, Igl W, Bailey JN, et al. A large genome-wide association study of age-related macular  
374 degeneration highlights contributions of rare and common variants. *Nat Genet.* 2016;48:134-143.
- 375 23. Burdon KP, Mitchell P, Lee A, et al. Association of open-angle glaucoma loci with incident  
376 glaucoma in the Blue Mountains Eye Study. *Am J Ophthalmol.* 2015;159:31-36.e31.
- 377 24. Dimasi DP, Burdon KP, Hewitt AW, et al. Genetic investigation into the endophenotypic status of  
378 central corneal thickness and optic disc parameters in relation to open-angle glaucoma. *Am J*  
379 *Ophthalmol.* 2012;154:833-842.e832.



- 380 25. Anderson CA, Pettersson FH, Clarke GM, Cardon LR, Morris AP, Zondervan KT. Data quality  
381 control in genetic case-control association studies. *Nat Protoc.* 2010;5:1564-1573.
- 382 26. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components  
383 analysis corrects for stratification in genome-wide association studies. *Nat Genet.* 2006;38:904-909.
- 384 27. Loh P-R, Danecek P, Palamara PF, et al. Reference-based phasing using the Haplotype Reference  
385 Consortium panel. *Nat Genet.* 2016;48:1443-1448.
- 386 28. Auton A, Abecasis GR, Altshuler DM, et al. A global reference for human genetic variation. *Nature.*  
387 2015;526:68-74.
- 388 29. Das S, Forer L, Schönherr S, et al. Next-generation genotype imputation service and methods. *Nat*  
389 *Genet.* 2016;48:1284-1287.
- 390 30. Chang CC, Chow CC, Tellier LCAM, Vattikuti S, Purcell SM, Lee JJ. Second-generation PLINK:  
391 rising to the challenge of larger and richer datasets. *Gigascience.* 2015;4:7.
- 392 31. Li X, Bykhovskaya Y, Haritunians T, et al. A genome-wide association study identifies a potential  
393 novel gene locus for keratoconus, one of the commonest causes for corneal transplantation in  
394 developed countries. *Hum Mol Genet.* 2012;21(2):421-429.
- 395 32. Fried LP, Borhani NO, Enright P, et al. The cardiovascular health study: Design and rationale. *Ann*  
396 *Epidemiol.* 1991;1:263-276.
- 397 33. Psaty BM, O'Donnell CJ, Gudnason V, et al. Cohorts for heart and aging research in genomic  
398 epidemiology (CHARGE) consortium: Design of prospective meta-analyses of genome-wide  
399 association studies from 5 cohorts. *Circ Cardiovasc Genet.* 2009;2:73-80.
- 400 34. Willer CJ, Li Y, Abecasis GR. METAL: Fast and efficient meta-analysis of genomewide association  
401 scans. *Bioinformatics.* 2010;26:2190-2191.
- 402 35. Ward LD, Kellis M. HaploReg: a resource for exploring chromatin states, conservation, and  
403 regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res.*  
404 2012;40:D930-934.
- 405 36. GTEx Consortium. The Genotype-Tissue Expression (GTEx) pilot analysis: Multitissue gene  
406 regulation in humans. *Science.* 2015;348:648-660.
- 407 37. Wagner AH, Anand VN, Wang WH, et al. Exon-level expression profiling of ocular tissues. *Exp Eye*  
408 *Res.* 2013;111:105-111.

- 409 38. You J, Corley SM, Wen L, et al. RNA-Seq analysis and comparison of corneal epithelium in  
410 keratoconus and myopia patients. *Sci Rep*. 2018;8:1-13.
- 411 39. Birch-Machin MA, Russell EV, Latimer JA. Mitochondrial DNA damage as a biomarker for  
412 ultraviolet radiation exposure and oxidative stress. *Br J Dermatol*. 2013;169 Suppl 2:9-14.
- 413 40. Afshari NA, Igo RP, Jr., Morris NJ, et al. Genome-wide association study identifies three novel loci  
414 in Fuchs endothelial corneal dystrophy. *Nat Commun*. 2017;8:14898.
- 415 41. Jurkunas U, Azar DT. Potential complications of ocular surgery in patients with coexistent  
416 keratoconus and Fuchs' endothelial dystrophy. *Ophthalmology*. 2006;113(12):2187-2197.
- 417 42. Vira S, Abugo U, Shih CY, et al. Descemet stripping endothelial keratoplasty for the treatment of  
418 combined fuchs corneal endothelial dystrophy and keratoconus. *Cornea*. 2014;33(1):1-5.

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420

421 **Figure 1:** Manhattan plot of association results in the discovery cohort. Results of logistic  
422 regression with the first three PCs as covariates ( $-\log_{10} p$ -values) are plotted for each chromosome.  
423 The red and blue lines represent the genome-wide significance threshold of  $p = 5 \times 10^{-8}$  and our  
424 threshold for follow-up of  $p = 10^{-6}$ , respectively.

425

426 **Table 1:** Demographics of the Australian discovery cohort and the three replication cohorts.  
427

Cohort	Cases			Controls		
	N	% male	Mean age [SD]	N	% male	Mean age [SD]
Discovery: Australia	522	56	45 [15.2]	655	47	65 [10.6]
Replication: USA	222	55	44 [13.3]	2927	39	72 [5.4]
Replication: AUS+NI	331	61	41 [15.9]	229	37	75 [11.5]
Replication: VIC	265	60	35 [14.9]	702	38	52 [15.2]

428

429 **Table 2:** Lead SNPs at all three loci with  $p < 1 \times 10^{-6}$  in discovery cohort.

430

Locus <sup>a</sup>	Chr	Lead SNP	Base pair <sup>b</sup>	A1/2	F_A <sup>c</sup>	F_U <sup>c</sup>	OR <sup>d</sup> [95% CI]	P	P (CCT) <sup>e</sup>
<i>PNPLA2</i>	11	rs61876744	820754	T/C	0.341	0.447	0.59 [0.49–0.71]	$7.46 \times 10^{-9}$	0.553
<i>MAML2</i>	11	rs10831500	95982642	G/T	0.422	0.330	1.59 [1.33–1.91]	$3.91 \times 10^{-7}$	0.014
<i>CSNK1E</i>	22	rs138380	38796629	G/A	0.384	0.524	0.49 [0.40–0.60]	$6.35 \times 10^{-12}$	0.790

431

432 <sup>a</sup>Locus assigned to the RefSeq protein-coding gene within or near the association signal interval.

433 <sup>b</sup>Genomic positions are based on hg19.

434 <sup>c</sup>F\_A, minor allele frequency in cases; F\_U, minor allele frequency in controls.

435 <sup>d</sup>Odds ratios with respect to A1.

436 <sup>e</sup>P-values in our previously reported analysis for CCT.<sup>19</sup>

437

438 **Table 3:** Replication and meta-analysis of association results at lead SNPs.

439

Locus <sup>a</sup>	Chr	Lead SNP <sup>b</sup>	Base pair <sup>c</sup>	A1/2	Discovery		Replication:USA		Replication: AUS+NI		Replication: VIC		Meta-analysis	
					OR [95% CI]	P	OR	P	OR [95% CI]	P	OR [95% CI]	P	P	direction
<i>PNPLA2</i>	11	rs61876744	820754	T/C	0.59 [0.49–0.71]	$7.46 \times 10^{-9}$	0.77 [0.63–0.95]	$8.88 \times 10^{-4}$	0.84 [0.65–1.08]	0.179	0.97 [0.79–1.19]	0.763	$2.45 \times 10^{-8}$	-----
<i>MAML2</i>	11	rs10831500	95982642	G/T	1.59 [1.33–1.91]	$3.91 \times 10^{-7}$	1.35 [1.11–1.65]	$5.69 \times 10^{-3}$	1.09 [0.85–1.40]	0.515	1.03 [0.84–1.26]	0.792	$3.83 \times 10^{-6}$	++++
<i>CSNK1E</i>	22	rs138378	38796159	A/G	0.50 [0.41–0.61]	$1.77 \times 10^{-11}$	0.95 [0.78–1.16]	0.790	0.97 [0.76–1.24]	0.818	0.93 [0.76–1.13]	0.448	$3.18 \times 10^{-4}$	-----

440

441 <sup>a</sup>Locus assigned to the RefSeq protein-coding gene within or near the association signal interval.

442 <sup>b</sup>The lead SNP at *CSNK1E* (rs138380) was not successfully genotyped in the AUS+NI and VIC cohorts, and a proxy (rs138378,  $R^2=0.938$ ) was used for replication  
443 instead.

444 <sup>c</sup>Genomic positions are based on hg19.

445

